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(54) Title: EXPRESSION-SECRETION VECTORS FOR THE PRODUCTION OF BIOLOGICALLY ACTIVE FV FRAGMENTS

(57) Abstract

Expression-secretion vectors capable of producing biologically active Fv fragments or single chain Fv molecules, host cells containing these expression-secretion vectors, and methods for producing biologically active Fv fragments or single chain Fv molecules.

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EXPRESSION-SECRETION VECTORS FOR THE PRODUCTION OF BIOLOGICALLY ACTIVE FV FRAGMENTS

BACKGROUND OF THE INVENTION

The Fv fragment is the smallest complete antigen binding site presently known. This fragment is composed of only the variable domains of the immunoglobulin variable heavy (V_H) and variable light (V_L) chains. The small size of the Fv fragment has generated a great deal of interest in the antibody and protein engineering fields because of its potential application in imaging, therapeutics, and structural studies. Initial attempts to generate Fv were made using proteolytic cleavage of whole antibody. However, this technique was hindered by difficulties in controlling both quality and yield (Inbar et al., Proc. Natl. Acad. Sci. USA 69, 2659 [1972]). Recombinant DNA techniques were later employed in attempts to express native Fv in bacterial cells. Some groups have tried to express the individual V_H and V_L chains and reassociate the chains *in vitro*. The production of these proteins intracellularly resulted in insoluble proteins which had to be denatured and renatured to generate functional antibody. It is not clear what the exposure to denaturants will do to native antibody structure

and hence these systems are less than ideal. More recently, the production of soluble native Fv (Skerra and Pluckthun, Science 240, 1038 [1988]) and other related fragments (Better et al., Science 240, 1041 [1988]) has been reported. However, the yields of native Fv reported in these systems are quite low (0.2 mg/liter of cells) for many practical applications (e.g., isotope labeling for 3-D NMR analysis).

It would be useful to have improved expression-secretion systems for the production of biologically active Fv fragments and single chain Fv molecules.

SUMMARY OF THE INVENTION

The present invention concerns expression-secretion systems for the production of biologically active Fv fragments and single chain Fv molecules.

In particular, the present invention concerns an expression-secretion vector capable of producing a biologically active Fv fragment comprising a DNA sequence encoding the T7 promoter, a DNA sequence encoding the variable domain of an immunoglobulin heavy chain (V_H), a DNA sequence encoding the variable domain of an immunoglobulin light chain (V_L), and one or more DNA sequences encoding one or more signal peptide sequences.

The present invention further concerns a host cell containing an expression-secretion vector capable of producing a biologically active Fv fragment comprising a DNA sequence encoding the T7 promoter, a DNA sequence encoding the variable domain of an immunoglobulin heavy chain, a DNA

sequence encoding the variable domain of an immunoglobulin light chain, and one or more DNA sequences encoding one or more signal peptide sequences.

The present invention additionally concerns a method for producing a biologically active Fv fragment comprising culturing a host cell containing an expression-secretion vector capable of producing a biologically active Fv fragment which comprises a DNA sequence encoding the T7 promoter, a DNA sequence encoding the variable domain of an immunoglobulin heavy chain, a DNA sequence encoding the variable domain of an immunoglobulin light chain, and one or more DNA sequences encoding one or more signal peptide sequences under conditions permitting expression-secretion of the biologically active Fv fragments.

The present invention also concerns an expression-secretion vector capable of producing a biologically active single chain Fv (sFv) molecule comprising a DNA sequence encoding the T7 promoter, a DNA sequence encoding a single chain Fv molecule, and a DNA sequence encoding a signal peptide sequence.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the nucleotide and deduced amino acid sequences of the (A) V_H [SEQ. ID NO. 1] and (B) V_L [SEQ. ID NO. 2] portions of antidigoxin monoclonal antibody 26-10. Restriction sites are shown.

Figure 2 shows the modified DNA sequences encoding and the deduced amino acid sequences of the signal peptide sequences (A) ompA [SEQ. ID NO. 3]

and (B) phoA [SEQ. ID NO. 4]. Arrows indicate the change of nucleotides at these particular positions to generate desirable restriction enzyme recognition sites.

Figure 3 shows the various plasmids produced in generating plasmid FvpD: (A) Construction of plasmid V_HpD; (B) Construction of plasmid V_LpD; (C) Construction of plasmid V_LpD-Xbal; (D) Construction of plasmid FvpD.

Figure 4 is a sodium dodecyl sulphate (SDS) polyacrylamide gel demonstrating the production of the 26-10 Fv fragment. Lanes 1-4: Eluted fraction numbers 3 to 6 from ouabain column. Peak of Fv is at fraction 3. Lane 5: Protein size standards 16·9, 14·4, 8·2 kd. Lane 6: Prestained protein size standards 110, 84, 47, 33, 24, 16 kd. Lane 7: Fv periplasmic fraction before column purification. Figure 5 shows the constructs of plasmid pT7PhoA 26-10sFv.

Figure 5 shows the construction of plasmid pT7PhoA26-10sFv.

Figure 6 shows the DNA sequence encoding and the amino acid sequence of the 26-10 single chain Fv molecule. Restriction sites and some 5' and 3' non-coding sequences are shown.

DETAILED DESCRIPTION OF THE INVENTION

The present invention concerns an expression-secretion vector capable of producing a biologically active Fv fragment comprising a DNA sequence encoding the T7 promoter, a DNA sequence encoding the variable domain of an immunoglobulin heavy chain, a DNA sequence encoding the variable domain of a immunoglobulin light chain, and one or more DNA sequences encoding one or more signal peptide

sequences. The present invention also concerns an expression-secretion vector capable of producing a biologically active single chain Fv (sFv) molecule comprising of DNA sequence encoding the T7 promoter, a DNA sequence encoding a single chain Fv molecule, and a DNA sequence encoding a signal peptide sequence. Preferably, the biologically active Fv fragment or sFv molecule has authentic N-termini (i.e., the mature Fv fragment or sFv molecule is generated by cleavage of the peptide bond between the carboxy terminus of the signal peptide sequence and the amino terminus of the variable domain of the immunoglobulin heavy or light chain). Further preferred are expression-secretion vectors wherein the signal peptide sequences are ompA and phoA. Additionally preferred are expression-secretion vectors wherein the DNA sequences encoding the signal peptide sequences have been modified to generate additional restriction enzyme sites without changing the amino acid sequences of the signal peptide sequences. Also preferred is an expression-secretion vector capable of producing a biologically active Fv fragment comprising a DNA sequence encoding the T7 promoter operatively linked to a DNA sequence encoding the variable domain of an immunoglobulin heavy chain, a DNA sequence encoding the variable domain of an immunoglobulin light chain, and one or more DNA sequences encoding one or more signal peptide sequences.

As used in this context, the term "operatively linked" means that the T7 promoter is capable of directing the transcription of the DNA

sequences encoding the variable domains of the immunoglobulin heavy and light chains.

As used in the present application, the term "Fv fragment" means the non-covalently associated variable domains of the immunoglobulin heavy and light chains which can bind antigen but which lack the effector functions of the constant regions of the immunoglobulin heavy and light chains.

As used in the present specification, the term "single chain Fv molecule" means a molecule in which variable domains of the immunoglobulin heavy and light chains which can bind antigen but which lack effector functions of the constant regions of the immunoglobulin heavy and light chains are joined using an amino acid linker.

As used in the present specification, the terms "biologically active Fv fragment" or "biologically active sFv molecule" means that the Fv fragment or sFv molecule is capable of specifically binding one or more of the same antigens as the full length antibody from which it is derived.

Expression-secretion vectors of utility in the present invention are often in the form of "plasmids", which refer to circular double stranded DNAs which, in their vector form, are not bound to the chromosome. However, the invention is intended to include such other forms of expression-secretion vectors which serve equivalent functions and which become known in the art subsequently hereto.

The expression-secretion vectors of the present invention capable of producing a biologically active Fv fragment at a minimum contain a DNA sequence encoding the T7 promoter, a

DNA sequence encoding the variable domain of an immunoglobulin heavy chain, a DNA sequence encoding the variable domain of an immunoglobulin light chain, one or more DNA sequences encoding one or more signal peptides sequences (e.g., ompA, phoA, pelB) and the remaining vector. The expression-secretion vectors of the present invention capable of producing a biologically active sFv molecule at a minimum contain a DNA sequence encoding a T7 promoter, a DNA sequence encoding a single chain Fv molecule, a DNA sequence encoding a signal peptide sequence and the remaining vector. ompA and phoA are signal peptide sequences which are encoded by DNA sequences identical to or derived from the *Escherichia coli* (*E. coli*) ompA and phoA loci. The ompA locus is the structural gene for an *E. coli* outer membrane protein, and the phoA locus is the structural gene of *E. coli* alkaline phosphatase. The remaining vector must, of course, contain an origin of replication, for example, a colEI origin of replication. The expression-secretion vectors may also include other DNA sequences known in the art, for example, stability leader sequences which provide for stability of the plasmid, transcription termination sequences, regulatory sequences which allow expression-secretion of the structural gene to be modulated (e.g., by the presence or absence of nutrients or other inducers in the growth medium), marker sequences (e.g., for ampicillin and kanamycin resistance) which are capable of providing phenotypic selection in transformed host cells, and sequences which provide sites for

cleavage by restriction endonucleases. The characteristics of the actual expression-secretion vector used must be compatible with the host cell which is to be employed. For example, when cloning in a bacterial system, the expression-secretion vector should contain DNA sequence (e.g., the T7 promoter) capable of functioning in that system. An expression-secretion vector as contemplated by the present invention is capable of directing the replication and the expression of DNA sequences encoding the variable domains of the immunoglobulin heavy and light chains or single chain Fv molecules.

Particularly preferred are the expression-secretion vectors designated FvpD or pT7PhoA 26-10sFv, described herein below, or expression-secretion vectors with the identifying characteristics of FvpD or pT7PhoA26-10sFv.

Suitable expression-secretion vectors containing the desired coding and control sequences may be constructed using standard recombinant DNA techniques known in the art, many of which are described in Maniatis, T. et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1982).

Of course, an integral component of the expression-secretion vectors of the present invention are DNA sequences coding for immunoglobulin V_H and V_L chains or for single chain Fv molecules. Such DNA sequences can be generated in various ways. In one approach, the DNA sequences of the present invention coding for immunoglobulin V_H and V_L chains or for single chain Fv molecules

can be chemically synthesized. For example, DNA sequences coding for immunoglobulin V_H and V_L chains or for single chain Fv molecules can be synthesized as a series of 100 base oligonucleotides that can then be sequentially ligated (via appropriate terminal restriction sites) so as to form the correct linear sequence of nucleotides [on the condition that the nucleotide sequences of the V_H and V_L chains or single chain Fv molecules are known].

In a second approach, DNA sequences coding for immunoglobulin V_H and V_L chains or for single chain Fv molecules can be generated using polymerase chain reaction (PCR). Briefly, pairs of synthetic DNA oligonucleotides at least 15 bases in length (PCR primers) that hybridize to opposite strands of the target (template) DNA sequence are used to enzymatically amplify the intervening region of DNA on the target sequence. Suitable template DNA sequences may be generated, for example, by isolating mRNA from a hybridoma of interest and reverse transcribing the mRNA. Suitable PCR primers may be chemically synthesized, and may be designed by sequencing mRNA from a hybridoma of interest, by sequencing the antibody molecule itself and producing degenerate primers, or by using generic primers [See, Orlandi et al., Proc. Natl. Acad. Sci. USA 86, 3833 (1989); Sastry et al., Proc. Natl. Acad. Sci. USA 86, 5728 (1989)]. Suitable 5' primers include, for example, those based on mature termini of the immunoglobulin V_H and V_L chains, and suitable 3' primers include, for example, those based on the heavy and light

chain J regions. Repeated cycles of heat denaturation of the template, annealing of the primers and extension of the 3'-termini of the annealed primers with a DNA polymerase results in amplification of the segment defined by the 5' ends of the PCR primers. See, U.S. Patent Nos. 4,683,195 and 4,683,202.

The present invention further concerns a host cell containing an expression-secretion vector capable of producing a biologically active Fv fragment comprising a DNA sequence encoding the T7 promoter, a DNA sequence encoding the variable domain of an immunoglobulin heavy chain, a DNA sequence encoding the variable domain of an immunoglobulin light chain, and one or more DNA sequences encoding one or more signal peptide sequences. The present invention also concerns a host cell containing an expression vector capable of producing a biologically active single chain Fv molecule comprising a DNA sequence encoding a T7 promoter, a DNA sequence encoding a single chain Fv molecule and a DNA sequence encoding a signal peptide sequence. Preferably, the biologically active Fv fragment or single chain Fv molecule has authentic N-termini (i.e., the mature Fv fragment or single chain Fv molecule is generated by cleavage of the peptide bond between the carboxy terminus of the signal peptide sequence and the amino terminus of the variable domain of the immunoglobulin heavy or light chain). Further preferred are expression-secretion vectors wherein the signal peptide sequences are ompA and phoA. Additionally preferred are expression-secretion

vectors wherein the DNA sequences encoding the signal peptide sequences have been modified to generate additional restriction enzyme sites without changing the amino acid sequences of the signal peptide sequences. Also preferred are host cells containing an expression-secretion vector capable of producing a biologically active Fv fragment comprising a DNA sequence encoding the T7 promoter operatively linked to a DNA sequence encoding the variable domain of an immunoglobulin heavy chain, a DNA sequence encoding the variable domain of an immunoglobulin light chain, and one or more DNA sequences encoding one or more signal peptide sequences.

Suitable host cells include *Escherichia coli* cells, such as *Escherichia coli* MC1061 cells. Other suitable *E. coli* strains include GM-1, SG-935 and 1023. Particularly preferred host cells are those containing an integrated copy of the T7 RNA polymerase gene, such as *E. coli* strains JM109/DE3 and BL21/DE3/pLysS.

The expression-secretion vectors of the present invention may be introduced into host cells by various methods known in the art. For example, transformation of host cells with expression-secretion vectors can be carried out as described in Maniatis et al., supra. However, other methods for introducing expression-secretion vectors into host cells, for example, electroporation, liposomal fusion, or viral or phage infection can also be employed.

Host cells producing active Fv fragments or single chain Fv molecules and which contain an

expression-secretion vector comprising a DNA sequence encoding the T7 promoter, a DNA sequence encoding the variable domain of an immunoglobulin heavy chain, a DNA sequence encoding the variable domain of an immunoglobulin light chain, and one or more DNA sequences encoding one or more signal peptide sequences, or which contain an expression-secretion vector comprising a DNA sequence encoding a T7 promoter, a DNA sequence encoding a single chain Fv molecule and a DNA sequence encoding a signal peptide sequence can be identified by one or more of the four general approaches: (a) DNA-DNA hybridization; (b) the presence or absence of marker gene functions; (c) assessing the level of transcription as measured by production of immunoglobulin V_H or V_L chain or single chain Fv molecule mRNA transcripts in the host cells; and (d) detection of the gene product biologically.

In the first approach, the presence of DNA sequences coding for immunoglobulin V_H or V_L chains or single chain Fv molecules can be detected by DNA-DNA or RNA-DNA hybridization using probes complementary to the DNA sequences.

In the second approach, the recombinant expression-secretion vector host system can be identified and selected based upon the presence or absence of certain marker gene functions (e.g., ampicillin and kanamycin resistance to antibiotics). A marker gene can be placed in the same plasmid as the DNA sequence coding for the immunoglobulin V_H or V_L chains or single chain Fv molecule under the regulation of the same or a different promoter used

to regulate the immunoglobulin V_H or V_L chain or single chain Fv molecule coding sequences. Expression of the marker gene can be used to select for cells harbouring the plasmid containing the DNA sequences coding for the immunoglobulin V_H or V_L chain or single chain Fv molecule.

In the third approach, the production of immunoglobulin V_H or V_L chain or single chain Fv molecule mRNA transcripts can be assessed by hybridization assays. For example, total RNA can be isolated and analyzed by Northern blotting or nuclease protection assay using a probe complementary to the RNA sequence.

In the fourth approach, the expression of immunoglobulin V_H or V_L chains or single chain Fv molecules can be assessed biologically, for example, by Western blotting or binding to antigen, or by sequencing of the protein product.

Once an expression-secretion vector has been introduced into an appropriate host cell, the host cell may be cultured under conditions permitting expression of large amounts of Fv fragments or single chain Fv molecules. Such Fv fragments or single chain Fv molecules may be used in the same manner as the full length antibody molecules from which they are derived. For example, they may be used for *in vivo* and *in vitro* immunological diagnostic procedures, and may be used therapeutically, either alone or after conjugation to drugs and toxins. They may also be used for structural studies, for example, using nuclear magnetic resonance (NMR) and X-ray crystallography.

If desired, the Fv fragments or single chain Fv molecules produced in this manner may be isolated and purified to some degree using various protein purification techniques. For example, chromatographic procedures such as ion exchange chromatography, gel filtration chromatography and immunoaffinity chromatography may be employed.

The DNA sequences of expression-secretion vectors, plasmids or DNA molecules of the present invention may be determined by various methods known in the art. For example, the dideoxy chain termination method as described in Sanger et al., Proc. Natl. Acad. Sci. USA 74, 5463-5467 (1977), or the Maxam- Gilbert method as described in Proc. Natl. Acad. Sci. USA 74, 560-564 (1977) may be employed.

It should be understood that the methodology described herein can be used to prepare Fv fragments or single chain Fv molecules derived from animal species other than mice, and Fv fragments or single chain Fv molecules for a wide variety of different antigens, for example, digoxin and fibrin. It should also be understood that the methodology described herein can be used in the production of modified Fv fragments or single chain Fv molecules. In this case, the DNA sequences coding for the variable domain of the immunoglobulin heavy chain, or the variable domain of the immunoglobulin light chain, or both, or for the single chain Fv molecule, can be modified (i.e., mutated) to prepare various mutations that change the amino acid sequence encoded by the mutated codon. These modified DNA

sequences may be prepared, for example, by mutating the DNA sequences coding for the variable domain of the immunoglobulin heavy chain, or the variable domain of the immunoglobulin light chain, or both, or for the single chain Fv molecule, so that the mutation results in the deletion, substitution, insertion, inversion or addition of one or more amino acids in the encoded polypeptide using various methods known in the art. For example, the methods of site-directed mutagenesis described in Taylor, J. W. et al., Nucl. Acids Res. 13, 8749-8764 (1985) and Kunkel, J. A., Proc. Natl. Acad. Sci. USA 82, 482-492 (1985) may be employed. In addition, kits for site-directed mutagenesis may be purchased from commercial vendors. For example, a kit for performing site-directed mutagenesis may be purchased from Amersham Corp. (Arlington Heights, IL). Contemplated modifications include, for example, humanization of Fv fragments derived from mice. See, Jones et al., Nature 321, 522 (1986). All such variations are included within the scope of the present invention.

As used above and elsewhere in the present application, the term "modified", when referring to a nucleotide or polypeptide sequence, means a nucleotide or polypeptide sequence which differs from the wild-type sequence found in nature.

The following examples are further illustrative of the present invention. These examples are not intended to limit the scope of the present invention, and provide further understanding of the invention.

Example 1

Cloning of Genes Encoding Antibody Fragments

Antidigoxin monoclonal antibody 26-10 is a high affinity (5×10^9 M^{-1}) antibody produced against digoxin conjugated to bovine serum albumin (Mudgett-Hunter et al. Mol. Immunol. 22, 477 [1985]). cDNA clones of the genes encoding the V_H and V_L portions of the 26-10 antibody were made by PCR amplification of cDNA generated by reverse transcription of mRNA isolated from the 2610 hybridoma and sequenced by the dideoxy chain termination method (Figures 1-A [SEQ. ID NO. 1] and 1-B [SEQ. ID NO. 2]). The DNA sequences were compared to genomic 2610 sequences [See, Near, R. I. et al., Mol. Immunol., 27, 901-909 (1990)] to verify that the authentic genes encoding 26-10 had been cloned.

Example 2

Construction of T7 Promoter Based Expression-Secretion Vector

A T7 promoter based expression-secretion vector was made through modification of the pT7-7 plasmid described in Tabor, S. et al., Proc. Natl. Acad. Sci. USA 82, 1074 (1985). The restriction sites in the polylinker region of pT7-7 were altered in such a way that convenient restriction sites were available for cloning DNA fragments containing both 26-10 V_H and V_L and their respective signal sequences (Figure 2 [SEQ. ID NO. 3 and SEQ. ID NO. 4]) on the same plasmid as an artificial operon. The signal sequences, ompA (Movva et al., J. Biol. Chem. 255, 27 [1980]) and

phoA (Inouye et al., J. Bacteriol. 149, 434 [1982]) were engineered such that novel restriction sites were generated without changing the amino acid sequence of the signal peptides. Hence, correct processing would be expected to generate the authentic N-terminal sequences of both chains and for the proteins to be secreted into the periplasmic space. These modified signal sequences were made by PCR using *E. coli* chromosomal DNA as template. For ompA, the following oligonucleotides were used as PCR primers:

5' Primer

5'-AACATATGAAAAAGACAGCTATCGCCATT-3' [SEQ. ID NO. 5]

3' Primer

5'-GAATTCGGCCTGCGCAACGGTCGCGAAACCAGCTAGCGCCACTGC-3' [SEQ. ID NO. 6].

For phoA, the following oligonucleotides were used as PCR primers:

5' Primer

5'-AACATATGAAACAAAGCACTATTGCACTGGCA-3' [SEQ. ID NO. 7]

3' Primer

5'-GAATTCGGCCTTGGTCACCGGGGTAAACAGTAA-3' [SEQ. ID NO. 8]

The modified signal sequences are shown in Figure 2 [SEQ. ID NO. 3 AND SEQ. ID NO. 4]. In both cases, and for all PCR procedures, PCR was performed using a GeneAmp Kit (Perkin-Elmer Cetus, Norwalk, CT) as recommended by the manufacturer.

The initial step in the construction of the expression-secretion vector was the previously mentioned modification of the pT7-7 polylinker region.

In order to generate a plasmid containing the 26-10 V_H region (see Figure 3A), the pT7-7

plasmid was cut with BamH1 and Sal1, then filled in with Klenow to effectively destroy the BamH1, Sal1 and Xba1 sites in the pT7-7 polylinker. The resulting vector was then cut with Xba1 and filled in with Klenow to destroy the Xba1 site upstream of the ribosome binding site. This plasmid was then cut with Sma1 and ligated using T4 DNA ligase with an Xba1 linker to generate the pT7-11 vector. The pT7-11 plasmid was cut with Nde1 and EcoR1 and ligated using T4 DNA ligase with an Nde1/EcoR1 fragment containing the ompA signal sequence, to generate the pT7-11 OmpA vector. The pT7-11 OmpA plasmid was then cut the Nru1 and EcoR1 and ligated using T4 DNA ligase in frame with a fragment encoding the V_H chain of 2610 generated by PCR using as template mRNA isolated from the 2610 hybridoma and the following oligonucleotide primers:

5' Primer

5'- AACATATGTTCGCGACCGTAGCGCAGGCCGAGGTCCAGCTGCAACAGTCCGGA-3'

[SEQ. ID NO. 9]

3' Primer

5'- TTGAATTCTTATTATGAGGAGACGGTGACTGAGGCTCC-3'

[SEQ. ID NO. 10]

Oligonucleotides used for the amplification generated Nru1 and EcoR1 sites at the 5' and 3' ends, respectively, of the amplified V_H fragment. The resulting plasmid was designated V_HpD.

In order to generate a plasmid containing the 26-10 V_L region (see Figure 3B), the phoA signal sequence was PCR amplified and cloned as a Nde1/EcoR1 fragment into unmodified pT7-7. The 26-10 V_L DNA was amplified by PCR using the oligonucleotide primers indicated below to generate

a fragment suitable for cloning into the pT7phoA expression-secretion vector:

5' Primer

5'-AACATATGACCAAGGCCGATGTTGTGATGACCCAAACTCCA-3'

[SEQ. ID NO. 11]

3' Primer

5'-TTCTGCAGTTATTACCGTTTGATTTCCAGCTTGGTGCC-3'

[SEQ. ID NO. 12]

The amplified 26-10 V_L fragment was cut with StyI and ligated using T4 DNA ligase with the pT7phoA plasmid which had been cut with StyI and SmaI, resulting in the ligation of the 26-10 V_L as a StyI/blunt ended fragment. The resulting plasmid was designated V_LpD.

The V_LpD plasmid was then cut with BamHI and SalI, filled in with Klenow and religated with T4 DNA ligase to generate plasmid V_LpD-XbaI (Figure 3C).

The V_HpD and V_LpD-XbaI plasmids were then used to construct the FvpD expression-secretion vector. The V_LpD-XbaI plasmid was cut with XbaI and HindIII to release the light chain containing the signal peptide sequence but lacking the T7 promoter sequence. This fragment was then ligated using T4 DNA ligase with XbaI/HindIII cut V_HpD to yield FvpD (Figure 3D).

The final construct (FvpD) as well as the intermediate constructs were sequenced by dideoxy DNA sequencing to assure no alterations affecting amino acid sequences had occurred during their construction/amplification.

Example 3

Expression of 26-10 Fv from Plasmid FvPD

To express 26-10 Fv from FvpD, a second compatible plasmid called Gp1-2 (Tabor et al., supra), which contains the T7 RNA polymerase gene under the control of the temperature sensitive lambda cI repressor and the kanamycin resistance gene, was co-transformed as described in Maniatis et al., supra. with FvpD into MC1061 *E. coli* cells using selection for both ampicillin and kanamycin resistant transformants. MC1061 cells may be obtained from Clontech (Palo Alto, CA) or the American Type Culture Collection (Rockville, MD). The transformants were inoculated into 2 x YT medium containing 20 µg/ml kanamycin and 50 µg/ml ampicillin, and grown at 25°C to OD_{600} = 2.0 prior to induction of 26-10 Fv. When the cells containing the Gp1-2 plasmid are shifted to 42°C for thirty minutes this inactivates the temperature sensitive repressor protein and permits expression of the T7 RNA polymerase gene. The T7 RNA polymerase protein is then able to promote transcription of the 26-10 V_H and V_L genes by utilizing the T7 promoter present upstream of the two genes. The cells were then shifted to 25°C for 30 minutes to facilitate the proper processing and assembly of the V_H and V_L polypeptides. As shown in Figure 4 (lane 7), temperature induced cells containing both the Gp1-2 and FvpD plasmids expressed two polypeptides that migrated with apparent molecular weights of 12 kd and 15 kd. The size of the 12 kd polypeptide was essentially identical to the size of the 26-10 V_L chain (12.2 kd) predicted from the

polypeptide encoded by the 26-10 V_L DNA sequence. The 15 kd polypeptide appeared to migrate slower than the predicted size (13.2 kd) of the polypeptide encoded by the 26-10 V_H DNA sequence. These two polypeptides also appeared to be properly localized as they were found to be greatly enriched in the periplasmic fraction. The 26-10 Fv was purified by affinity chromatography of the periplasmic fraction on a ouabain-Sepharose affinity column (ouabain is a digoxin congener). The periplasmic fraction was harvested by osmotic shock as described in Skerra et al., Science 240, 1038 (1988). All steps were performed on ice or at 4°C. After induction, the cells from a 1 liter culture were harvested by centrifugation at 4000 x g for 10 minutes. The cell pellet was suspended in 10 ml of TES buffer (0.2 M Tris HCl pH 8.0, 0.5 mM EDTA, 0.5 M sucrose). The suspended cells were then subjected to osmotic shock by the addition of 15 mls of diluted TES (TES diluted 1:4 with H_2O) to release the proteins present in the periplasmic space. After a 30 minute incubation on ice, the cells were removed by successive centrifugations of 5000 x g for 10 minutes and 38,000 g for 15 minutes. The supernatant containing the periplasmic fraction was then subjected to affinity chromatography. Upon elution of the bound material with 20 mM ouabain, fractions 3 and 4 (Figure 4, lanes 1 and 2) revealed two polypeptides of the correct size that were selectively purified. The following evidence indicated that this is 26-10 Fv: (1) It competed with ^{125}I-26-10 whole antibody in competitive RIA assays; (2) It bound ^{125}I-digoxin

with K_A=1.3 x 10^9 M^{-1} compared to 5 x 10^9 M^{-1} for 26-10 whole antibody; (3) N-terminal sequencing of V_L and V_H variable domains indicated correct processing and expected N-terminal sequences. The yield of purified Fv was demonstrated to be 1 mg/L. It should be possible to further improve the yield by using protease deficient strains as host, by optimizing fermentation conditions, by using alternative signal sequences, and by co-expressing enzymes and chaperones (e.g., heavy chain binding protein [BIP]) that are normally employed for immunoglobulin chain assembly in mammalian cells.

The 26-10 Fv made by this method is stable for at least a two months, and probably longer, when stored at 4°C at nM range protein concentrations.

Example 4

Alternate Expression of 26-10 Fv from FvpD

A second method was also used to express 26-10 Fv from FvpD. In this method, the FvpD plasmid was transformed into *E. coli* strain JM109/DE3 [Promega; See also, Studier, F.W. et al., Methods in Enzymology 185, 60-88 (ed. D.V. Goeddel) Academic Press (1990)]. JM109/DE3 contains an integrated copy of the T7 RNA polymerase gene under the control of a lac promoter. JM109/DE3 cells harboring the FvpD plasmid were grown until the A600nm of the cells measured between 1.0 and 2.4 in modified 2 x YT medium (2% bacto tryptone, 1% yeast extract, 0.5% sodium chloride, 0.2% glycerol, 50 mM potassium phosphate pH 7.2) with glucose (0.4%), ampicillin (50 mg/liter) at 37°C. The cells were then cooled to 24°C. Subsequently, isopropyl

beta-D-thiogalactoside (IPTG) was added to a final concentration of 0.05 mM to induce transcription of the T7 RNA polymerase gene. After the addition of IPTG, the cells were allowed to incubate at 24°C for 16 hours, and screened for periplasimic proteins.

It was found that osmotic shock supernatants (the periplasmic fractions from IPTG-treated JM109/DE3 cells containing the FvpD plasmid) contained two proteins that comigrated with proteins found in heat-treated MC1061/Gp1-2 cells. These two polypeptides appeared to be greatly enriched in the osmotic shock supernatant. When 26-10 Fv was purified from the osmotic shock supernatant using a ouabain-Sepharose column (see Example 3), two polypeptides were isolated of the approximate sizes expected for the 26-10 V_H and V_L chains (15 and 12 kD). The yield of affinity purified 26-10 Fv from the JM109/DE3 strain was 14 mg/liter. In the JM109/DE3 strain, the maximum level of 26-10 Fv accumulation was observed approximately 16 hours after induction, while in MC1061/GP1-2 (Example 3), the maximum level of 26-10 Fv accumulation occurred one hour after the start of induction. Coomassie staining of protein fractions separated by SDS-PAGE indicated that in both JM109/DE3 and MC1061/Gp1-2 (Example 3), most of the 26-10 Fv protein was found in the periplasm. N-terminal protein sequence analysis of the JM109/DE3 produced protein revealed that both the V_H (approximately 15 kD) and V_L (12 kD) chains had been correctly processed by the bacterial export system. In both

the JM109/DE3 and MC1061/Gp1-2 (Example 3) strains, it was necessary to cool the cells to 25°C after the protein inductions. Incubation at temperatures exceeding 27°C resulted in the accumulation of proteins of approximately 17 kD and 12 kD that did not bind to the ouabain-Sepharose column.

Example 5

Expression of Biologically Active Single Chain Fv

The expression systems of the present invention were also used to express biologically active single chain Fv (sFv) molecules.

The sFv form of the 26-10 antibody was constructed by PCR amplification with mutagenic oligonucleotides to create novel restriction sites (and to insert sequences encoding a peptide linker between the two chains.) Briefly, as summarized in Figure 5, the genes encoding the variable regions of the light (V_L) and heavy (V_H) chains were separately PCR amplified under the conditions described in Example 2 using as a template the cDNA clones of the genes encoding the V_H and V_L portions of the 26-10 antibody (see Example 1) and the following oligonucleotide primers:

3' V_L 26-10 Sequence Overlap Extension (SOE)

5'-AGAGCCGGATCCACCGGAACCGGAGCCGCCAGAACCAGAACCACCCCGTTTGATTTC CAGCTTGGT-3'

[SEQ. ID NO. 13]

5' V_L 26-10 BstE2 (for PhoA pT7)

5'-CCATCGGTGACCAAAGCCGATGTTGTGATGACCCAAACT-3'

[SEQ. ID NO. 14]

5' V_H 26-10 SOE

5'-GGTGGTTCTGGTTCTGGCGGCTCCGGTTCCGGTGGATCCGGCTCTGAGGTCCAGCTG CAACAGTCC-3'

[SEQ. ID NO. 15]

3' V_H 26-10 SalI
5'-CCCGTCGACCTGCAGGCATGCGGATCCTTATGAGGAGACGGTGACTGAGGCTCC-3'
[SEQ. ID NO. 16]

These oligonucleotides had complementary sequences, and included sequences encoding the peptide linker engineered between the V_L and V_H chains, so that the V_L and V_H sequences could later, after a second round of PCR amplification, form a complete double stranded DNA molecule encoding a single chain Fv molecule containing a 15 amino acid linker with the following sequence:

-Gly-Gly-Ser-Gly-Ser-Gly-Gly-Ser-Gly-Ser-Gly-Gly-Ser-Gly-Ser
[SEQ. ID NO. 17]

This DNA construct was designated PCR amplified 26-10 sFv. The PCR amplified 26-10 Fv and the plasmid designated pT7PhoA (See Example 2) were both cut with the restriction enzymes BstE2 and SalI and ligated, resulting in the plasmid designated pT7PhoA26-10sFv. This plasmid encodes a single chain protein with the following domains (going from the N-terminus to the C-terminus: PhoA leader - 26-10 variable light chain- linker- 26-10 variable heavy chain) (See Figure 6 [SEQ. ID NO. 18] for the DNA and encoded amino acids sequences of this construct).

The pT7PhoA26-10sFv plasmid was transformed by the $CaCl_2$ method (See, Maniatis et al., supra) into E. coli strain BL21/DE3/pLysS [See, Studier, F.W. et al., Methods in Enzymology 185, 60-88 (ed. D.V. Goeddel) Academic Press (1990)]. Cells harboring the pT7PhoA26-10sFv plasmid were grown overnight in Minimal Medium (7.6 mM NH_4SO_4,

11.0 mM sodium acetate, 12.7 mM succinic acid, 60.3 mM K_2HPO_4, 68 μM $CaCl_2.2H_2O$, 35 μM $ZnSO_4.7H_2O$, 59 μM $MnSO_4.H_2O$, 741 μM thiamin, 2032 μM niacin, 12 μM biotin, 40 μM $FeCl_3.6H_2O$, 3 μmM $Na_2MoO_4.2H_2O$, 3 μM $CuSO_4.5H_2O$, 3 μM H_3BO_3, 3 μM vitamin B-12, 4 mM $MgSO_4$, 22 mM glucose, 50 μg/ml ampicillin, 20 μg/ml chloramphenicol) at 37°C, then diluted 1:20 into 2 x YT medium (as in Example 4) at 37°C and grown until the A_{600nm} of the cells measured between 0.5 and 1.0. The cells were then cooled to 24°C. Subsequently IPTG was added to a final concentration of 0.2 mM to induce transcription of the T7 RNA polymerase gene. After the addition of IPTG the cells were allowed to incubate at 24°C for 16 hours and screened for periplasmic proteins and proteins in the culture supernatant. Under these conditions, a yield of 3 to 10 mg of affinity purified 26-10 sFv protein per liter cell culture was obtained. The affinity purified material had a molecular weight of about 29 kd, as shown by SDS-PAGE, which is in good agreement with the theoretically predicted molecular weight of about 26 kD. The fact the 26-10 sFv protein is biologically active was shown by its ability to bind to and be purified using a ouabain-Sepharose affinity column (see Example 3).

All publications and patents referred to in the present application are incorporated herein by reference to the same extent as if each individual publication or patent was specifically and individually indicated to be incorporated by reference.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANTS: Ng, Shi Chung; Anthony, James G.; Wong, Sui-Lam

(ii) TITLE OF THE INVENTION: Expression-Secretion Vectors for the Production of Biologically Active Fv Fragments

(iii) NUMBER OF SEQUENCES: 12

(iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: Burton Rodney - Squibb Corporation

(B) STREET: P.O. Box 4000

(C) CITY: Princeton

(D) STATE: New Jersey

(E) COUNTRY: USA

(F) ZIP: 08543-4000

(2) INFORMATION FOR SEQ. ID NO.: 1

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 357 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double stranded

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ. ID NO.:1

```
GAG GTC CAG CTG CAA CAG TCC GGA CCT GAG CTG GTG AAG CCT GGG GCT  48
Glu Val Gln Leu Gln Gln Ser Gly Pro Glu Leu Val Lys Pro Gly Ala
 1               5                  10                  15

TCA GTG AGG ATG TCC TGC AAG TCT TCT GGA TAC ATA TTC ACT GAC TTC  96
Ser Val Arg Met Ser Cys Lys Ser Ser Gly Tyr Ile Phe Thr Asp Phe
            20                  25                  30

TAC ATG AAC TGG GTG AGG CAG AGC CAT GGA AAG AGC CTT GAT TAC ATT 144
Tyr Met Asn Trp Val Arg Gln Ser His Gly Lys Ser Leu Asp Tyr Ile
        35                  40                  45

GGA TAT ATT TCT CCT TAC AGT GGT GTT ACT GGC TAC AAC CAG AAG TTC 192
Gly Tyr Ile Ser Pro Tyr Ser Gly Val Thr Gly Tyr Asn Gln Lys Phe
    50                  55                  60

AAA GGC AAG GCC ACA TTG ACT GTA GAC AAG TCC TCC AGC ACA GCC TAC 240
Lys Gly Lys Ala Thr Leu Thr Val Asp Lys Ser Ser Ser Thr Ala Tyr
65                  70                  75                  80

ATG GAG CTC CGC AGC CTG ACA TCG GAG GAT TCT GCA GTC TAT TAC TGT 288
Met Glu Leu Arg Ser Leu Thr Ser Glu Asp Ser Ala Val Tyr Tyr Cys
                85                  90                  95

GCA GGA TCG TCG GGG AAT AAG TGG GCT ATG GAC TAC TGG GGT CAC GGA 336
Ala Gly Ser Ser Gly Asn Lys Trp Ala Met Asp Tyr Trp Gly His Gly
            100                 105                 110

GCC TCA GTC ACC GTC TCC TCA                                     357
Ala Ser Val Thr Val Ser Ser
        115
```

(2) INFORMATION FOR SEQ. ID NO.: 2
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 339 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double stranded
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ. ID NO.: 2

```
GAT GTT GTG ATG ACC CAA ACT CCA CTC TCC CTG CCT GTC AGT CTT GGA  48
Asp Val Val Met Thr Gln Thr Pro Leu Ser Leu Pro Val Ser Leu Gly
 1               5                  10                  15

GAT CAA GCC TCC ATC TCT TGC AGA TCT AGT CAG AGC CTT GTA CAC AGT  96
Asp Gln Ala Ser Ile Ser Cys Arg Ser Ser Gln Ser Leu Val His Ser
            20                  25                  30

AAT GGA AAT ACC TAT TTA AAT TGG TAC CTG CAG AAG GCA GGC CAG TCT 144
Asn Gly Asn Thr Tyr Leu Asn Trp Tyr Leu Gln Lys Ala Gly Gln Ser
        35                  40                  45

CCA AAG CTC CTG ATC TAC AAA GTT TCC AAC CGA TTT TCT GGG GTC CCA 192
Pro Lys Leu Leu Ile Tyr Lys Val Ser Asn Arg Phe Ser Gly Val Pro
    50                  55                  60

GAC AGG TTC AGT GGC AGT GGA TCA GGG ACA GAT TTC ACA CTC AAG ATC 240
Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Lys Ile
65                  70                  75                  80

AGC AGA GTG GAG GCT GAA GAT CTG GGA ATT TAT TTC TGC TCT CAA ACT 288
Ser Arg Val Glu Ala Glu Asp Leu Gly Ile Tyr Phe Cys Ser Gln Thr
                85                  90                  95

ACA CAT GTT CCT CCG ACG TTC GGT GGA GGC ACC AAG CTG GAA ATC AAA 336
Thr His Val Pro Pro Thr Phe Gly Gly Gly Thr Lys Leu Glu Ile Lys
            100                 105                 110

CGG                                                             339
Arg
```

(2) INFORMATION FOR SEQ. ID NO.: 3
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 68 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double stranded
(D) TOPOLOGY: circular

(xi) SEQUENCE DESCRIPTION: SEQ. ID NO.: 3

```
AACAT ATG AAA AAG ACA GCT ATC GCC ATT GCA GTG GCG CTA GCT GGT    47
      Met Lys Lys Thr Ala Ile Ala Ile Ala Val Ala Leu Ala Gly
        1               5                  10

                                                                68
TTC GCG ACC GTT GCG CAG GCC
Phe Ala Thr Val Ala Gln Ala
 15                  20
```

(2) INFORMATION FOR SEQ. ID NO.: 4
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 68 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double stranded
(D) TOPOLOGY: circular

(xi) SEQUENCE DESCRIPTION: SEQ. ID NO.: 4

```
AACAT ATG AAA CAA AGC ACT ATT GCA CTG GCA CTC TTA CCG TTA CTG    47
      Met Lys Gln Ser Thr Ile Ala Leu Ala Leu Leu Pro Leu Leu
        1               5                  10

                                                                68
TTT ACC CCG GTG ACC AAG GCC
Phe Thr Pro Val Thr Lys Ala
 15                  20
```

(2) INFORMATION FOR SEQ. ID NO.: 5
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single stranded
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ. ID NO.: 5

```
AACATATGAA AAAGACAGCT ATCGCCATT                                 29
```

(2) INFORMATION FOR SEQ. ID NO.: 6
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 45 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single stranded
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ. ID NO.: 6

GAATTCGGCC TGCGCAACGG TCGCGAAACC AGCTAGCGCC ACTGC 45

(2) INFORMATION FOR SEQ. ID NO.: 7
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single stranded
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ. ID NO.: 7

AACATATGAA ACAAAGCACT ATTGCACTGG CA 32

(2) INFORMATION FOR SEQ. ID NO.: 8
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single stranded
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ. ID NO.: 8

GAATTCGGCC TTGGTCACCG GGGTAAACAG TAA 33

(2) INFORMATION FOR SEQ. ID NO.: 9
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 53 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single stranded
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ. ID NO.: 9

AACATATGTT CGCGACCGTA GCGCAGGCCG AGGTCCAGCT GCAACAGTCC GGA 53

(2) INFORMATION FOR SEQ. ID NO.: 10
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 38 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single stranded
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ. ID NO.: 10

TTGAATTCTT ATTATGAGGA GACGGTGACT GAGGCTCC 38

(2) INFORMATION FOR SEQ. ID NO.: 11
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 41 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single stranded
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ. ID NO.: 11

AACATATGAC CAAGGCCGAT GTTGTGATGA CCCAAACTCC A 41

(2) INFORMATION FOR SEQ. ID NO.: 12
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 38 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single stranded
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ. ID NO.: 12

TTCTGCAGTT ATTACCGTTT GATTTCCAGC TTGGTGCC 38

What is Claimed is:

1. An expression-secretion vector capable of producing a biologically active single chain Fv molecule comprising a DNA sequence encoding the T7 promoter, a DNA sequence encoding a single chain Fv molecule, and a DNA sequence encoding a signal peptide sequence.

2. The expression-secretion vector according to Claim 1 wherein the signal peptide sequence is phoA.

3. A host cell comprising an expression-secretion vector according to Claims 1 or 2.

4. The host cell according to Claim 3 wherein the host cell contains a stably integrated copy of the T7 RNA polymerase gene.

5. The host cell according to Claim 4 wherein the host cell is *E. coli* strain JM109/DE3 or *E. coli* strain BL21/DE3/pLysS.

6. A method for producing a biologically active single chain Fv molecule comprising culturing a host cell according to Claim 3 under conditions permitting expresison of the biologically active single chain Fv molecule.

FIGURE 1-A

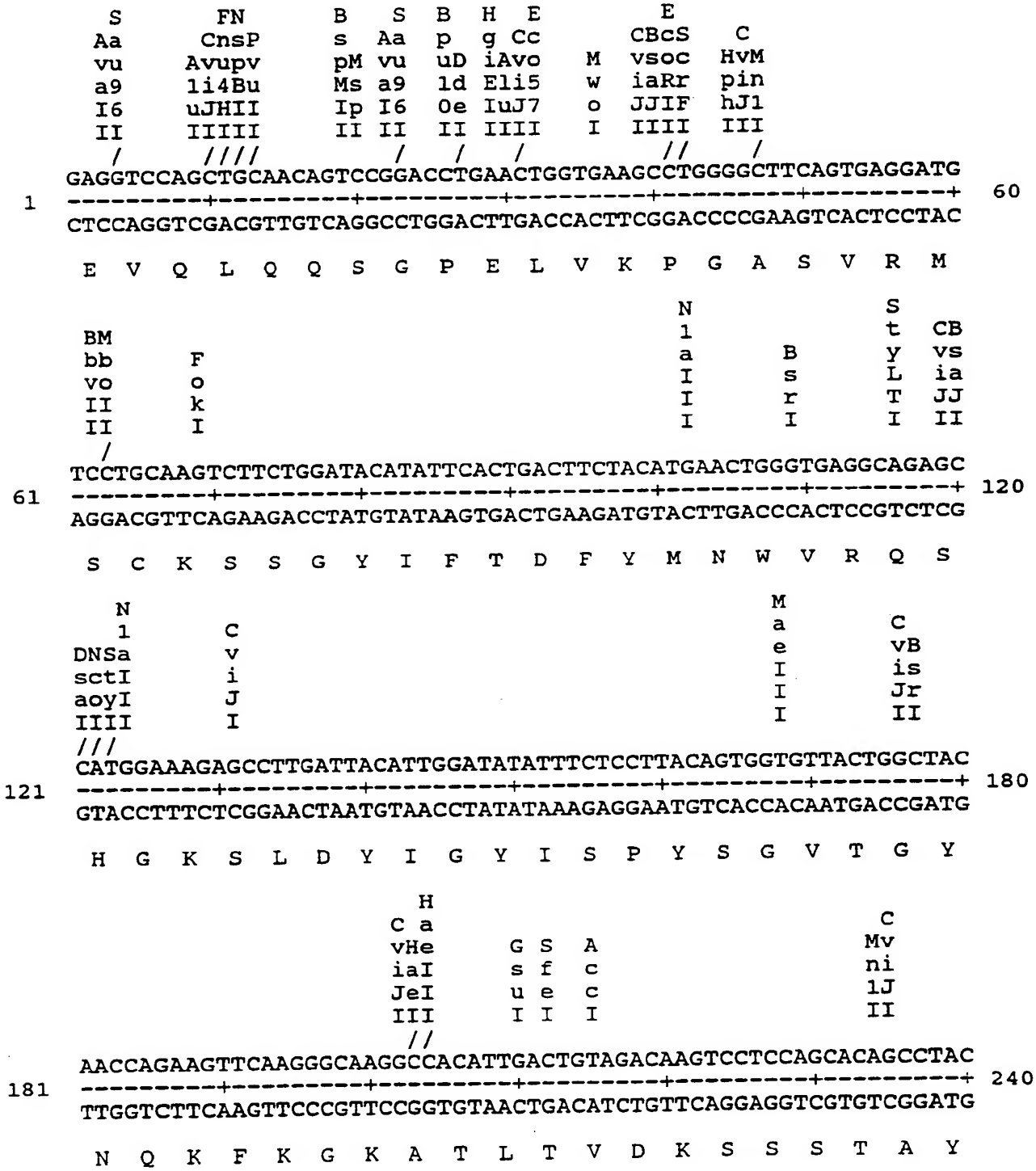


FIGURE 1-A (continued)

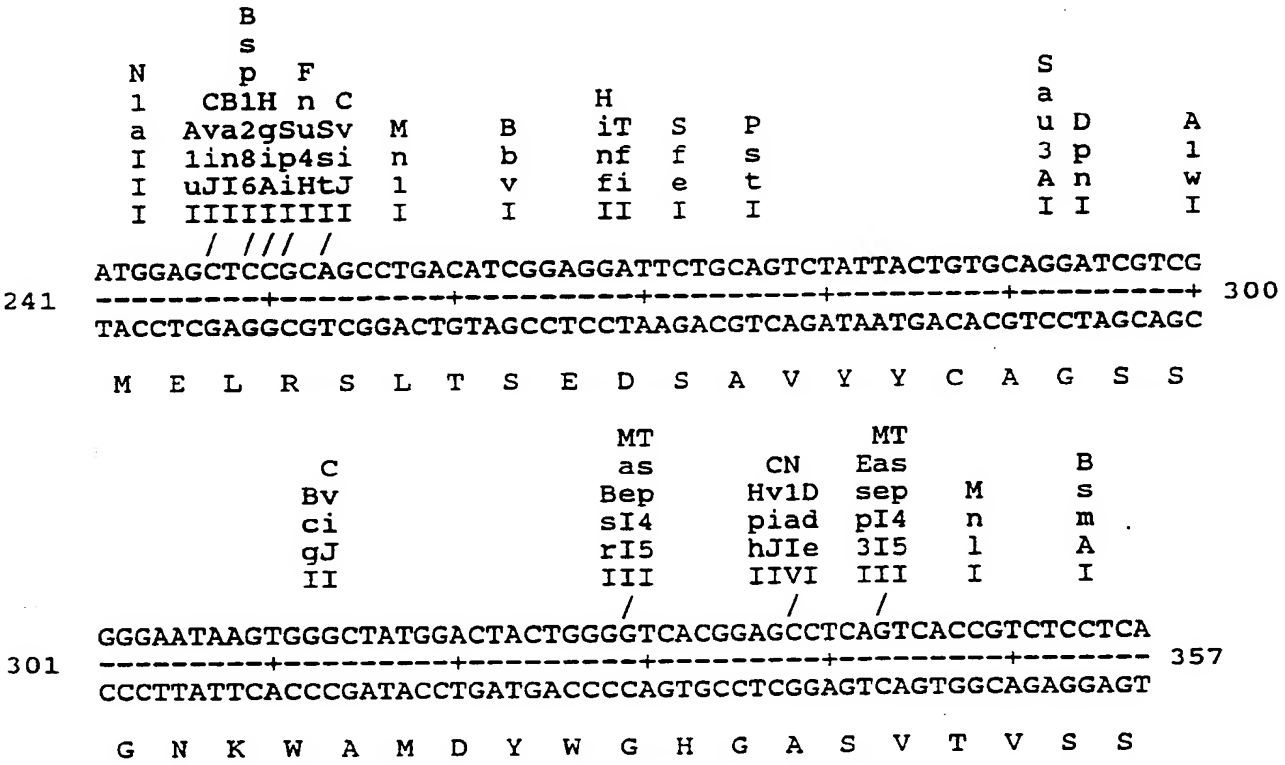


FIGURE 1-B

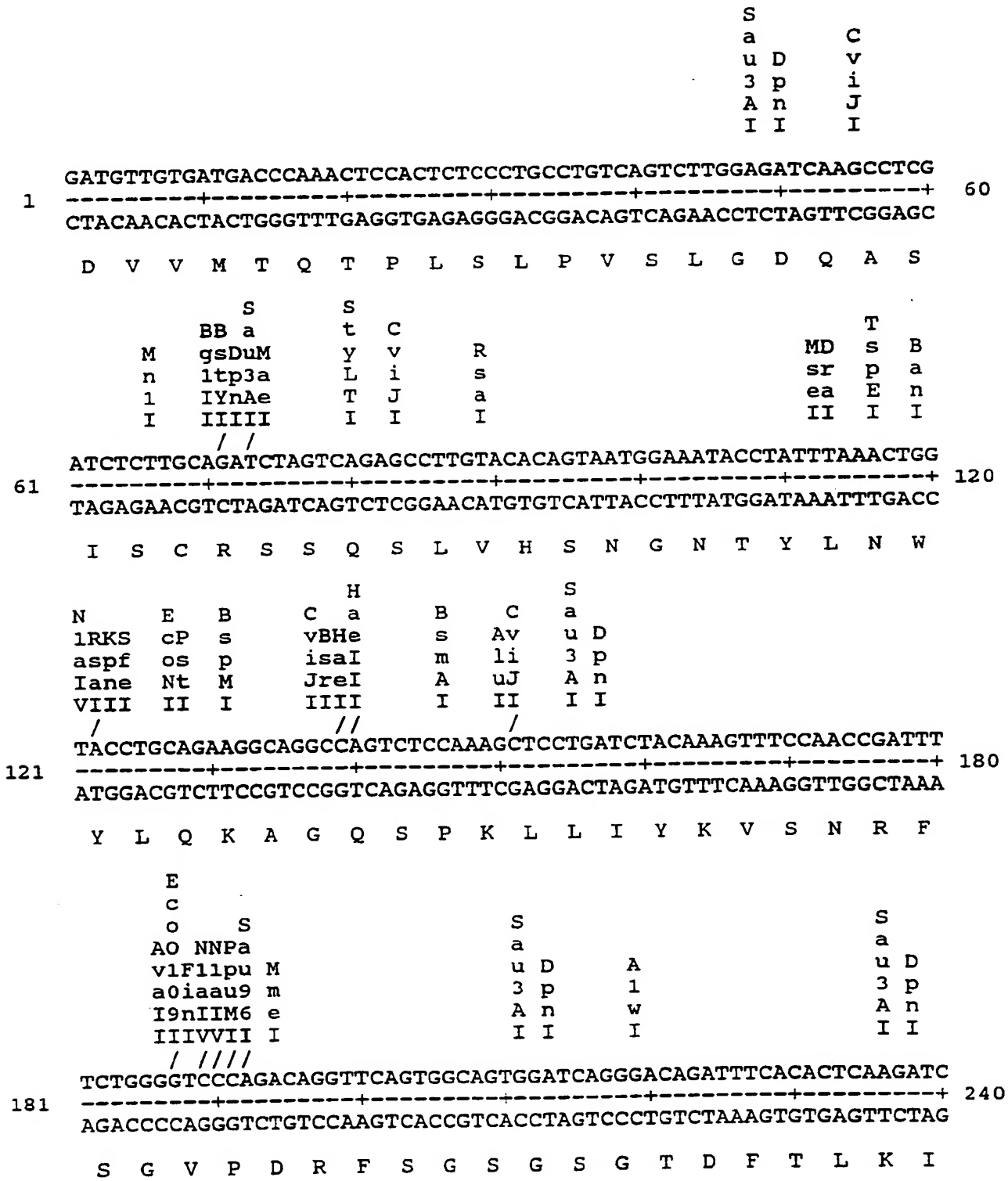
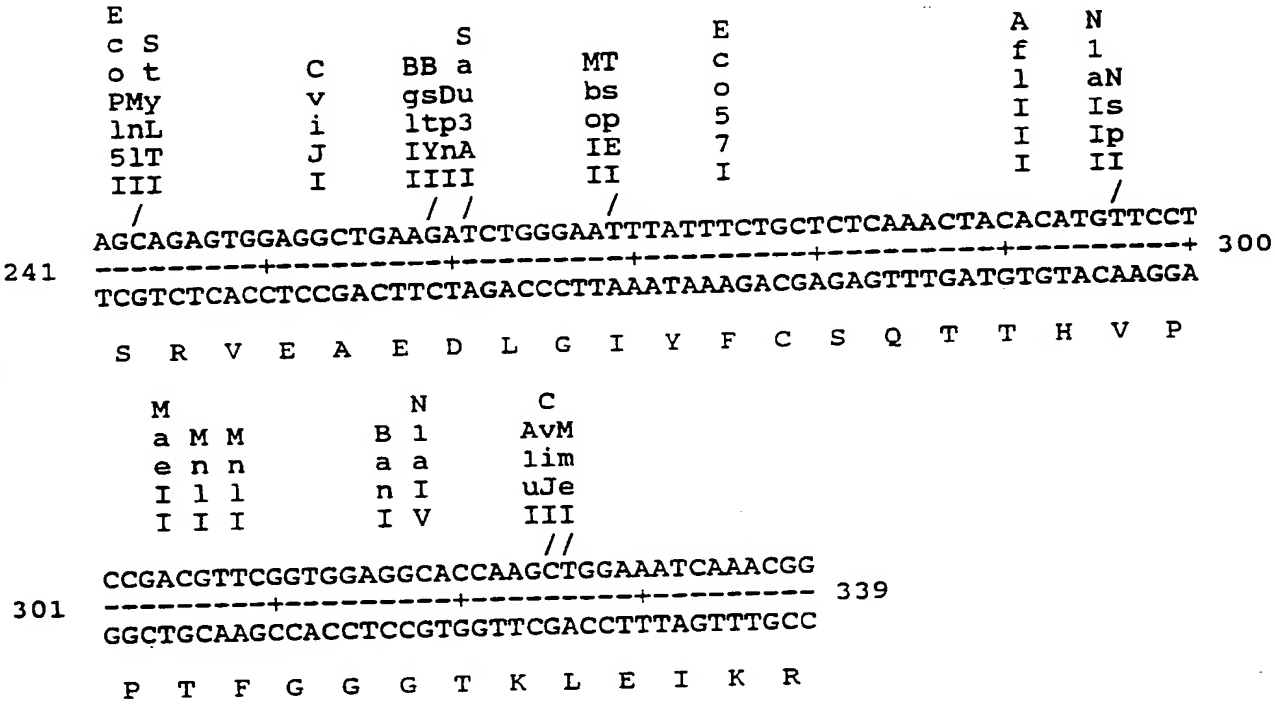


FIGURE 1-B (continued)



SUBSTITUTE SHEET

FIGURE 2A

Omp A:

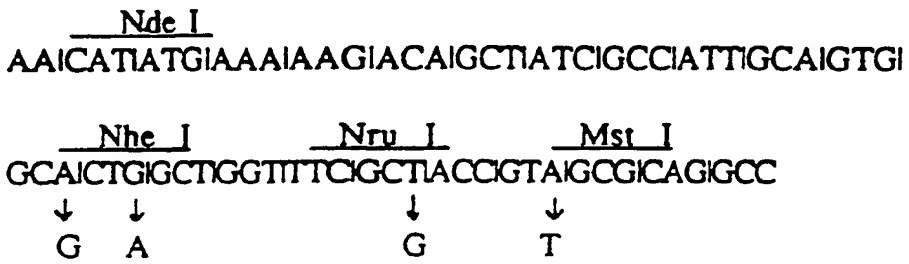


FIGURE 2B

Pho A:

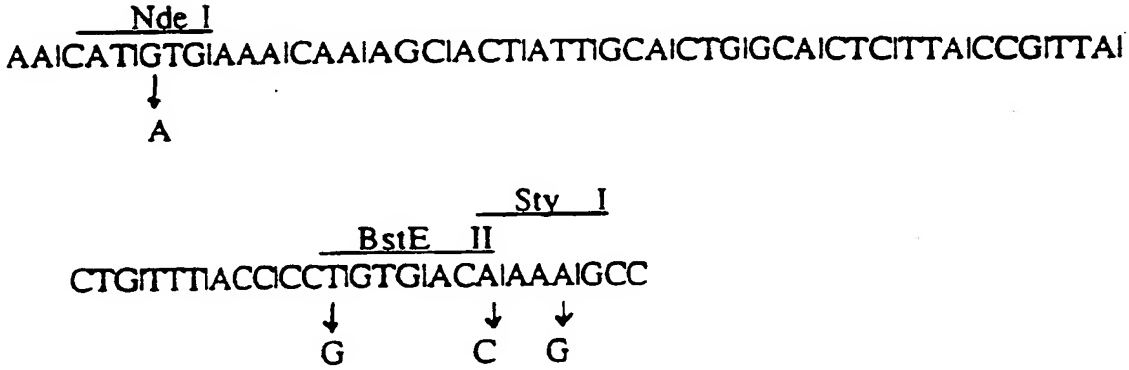


FIGURE 3-A

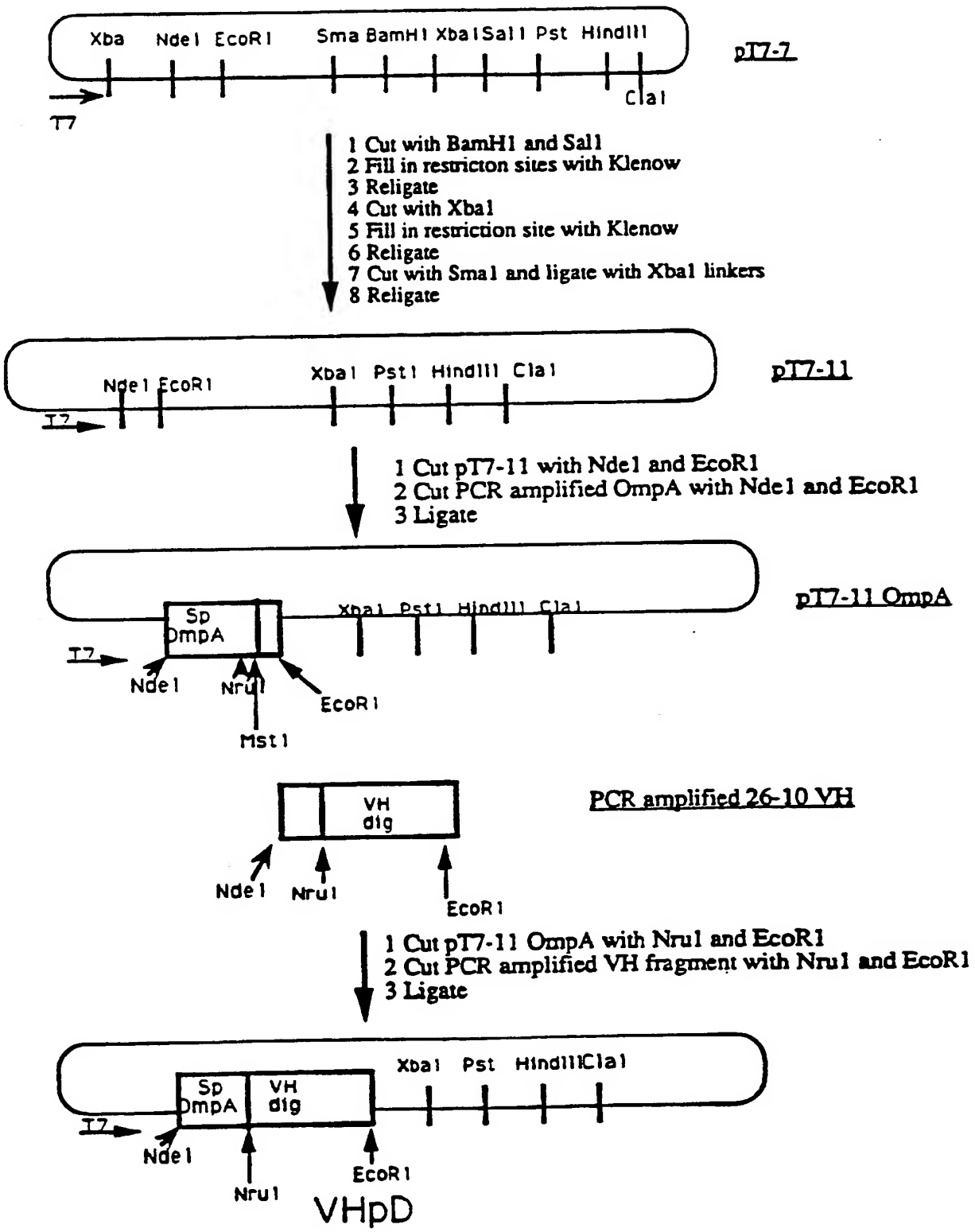


FIGURE 3-B

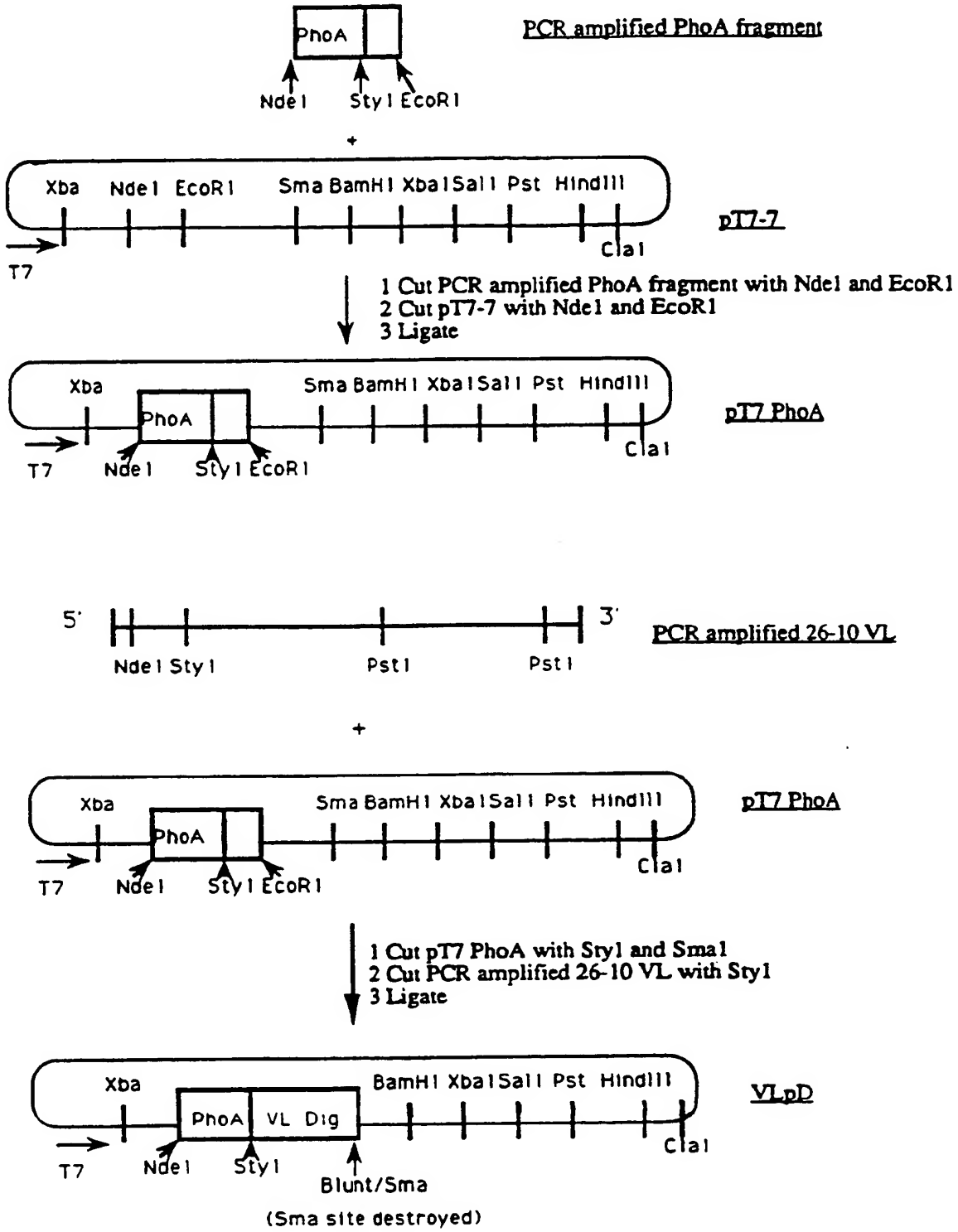


FIGURE 3-C

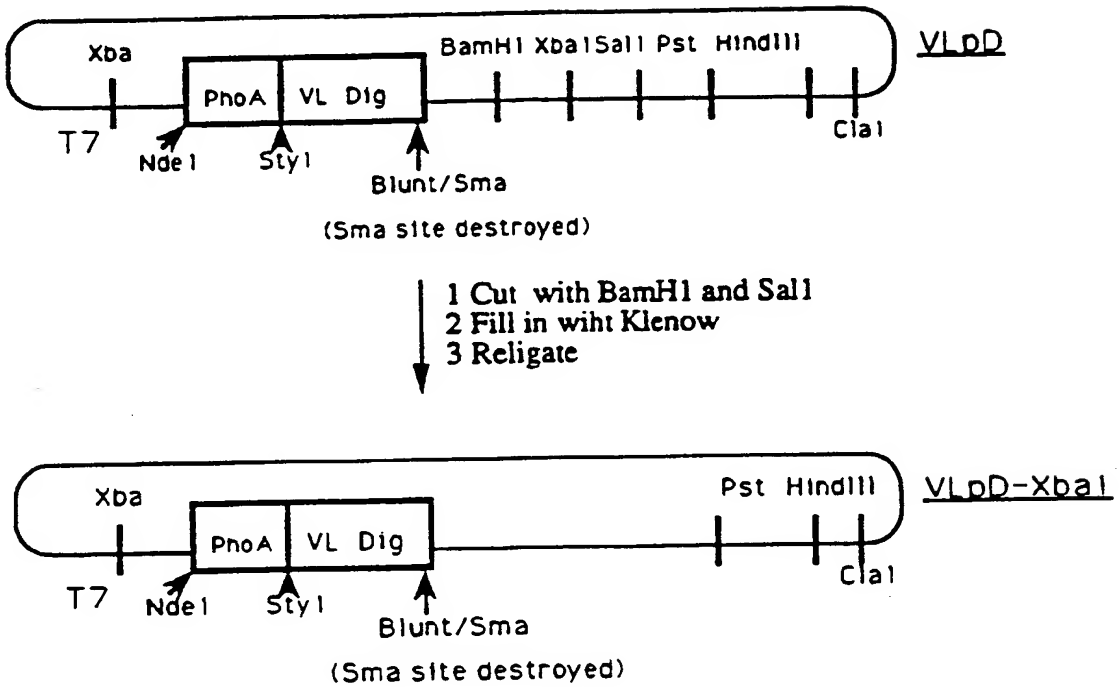
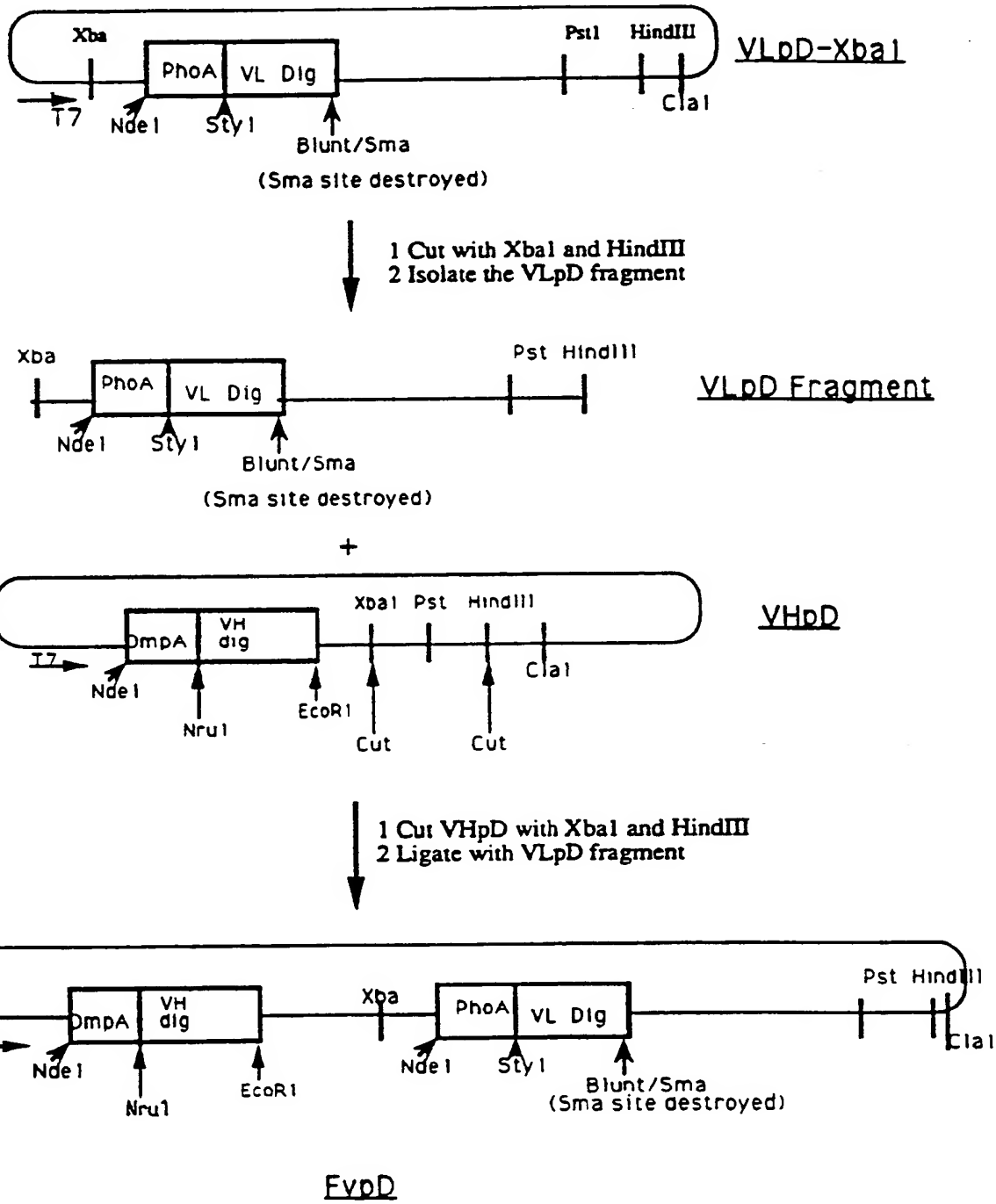


FIGURE 3-D



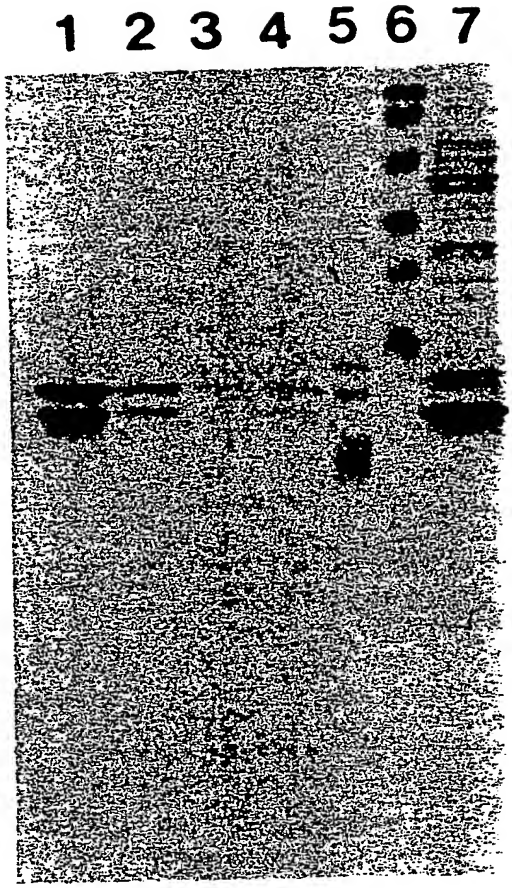


FIG. 4

FIGURE 5

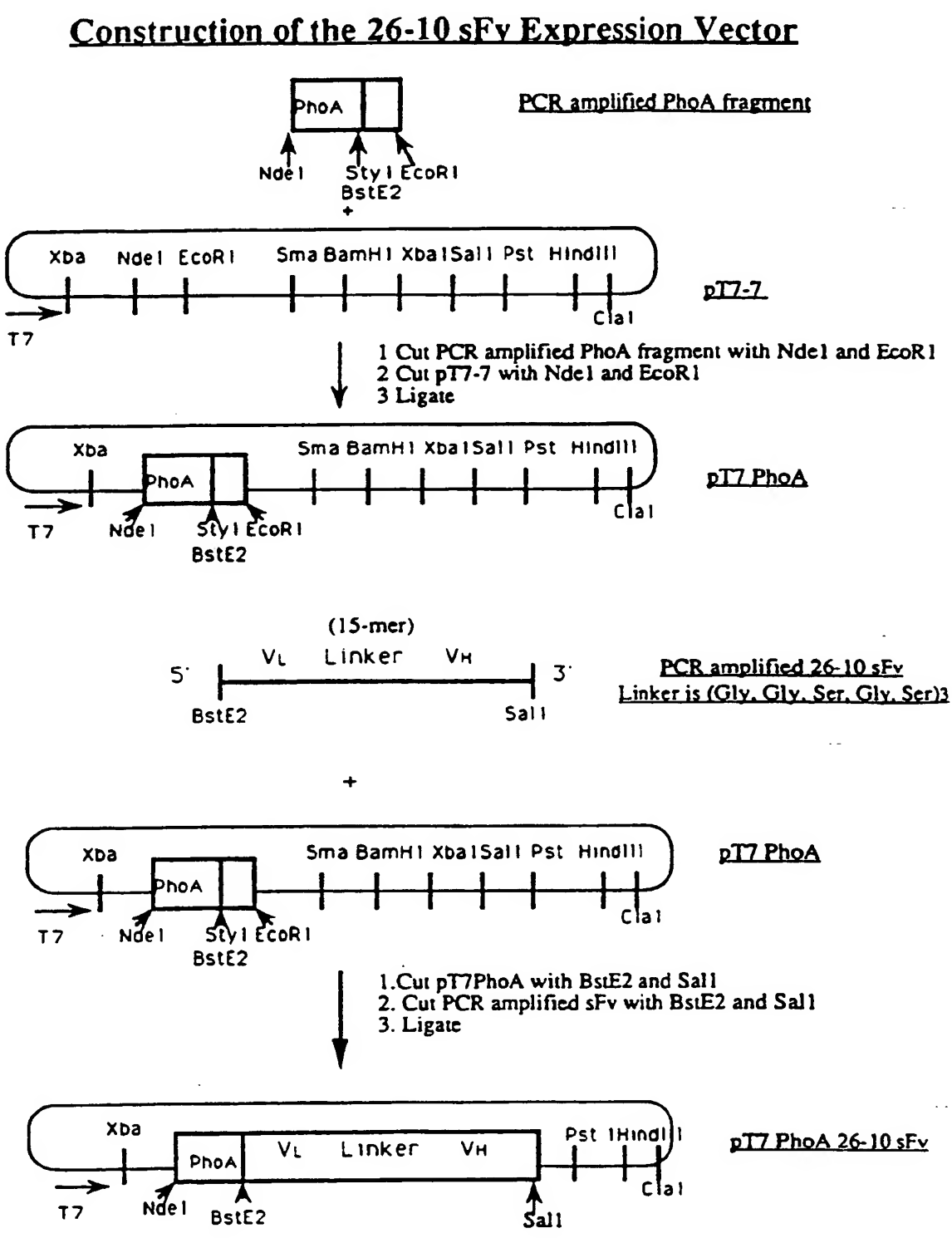


FIGURE 6

(Linear) MAP of: 2610 sFv from: 1 to: 848

	Nucleotides	Amino Acids
phoA signal seq	6-80	1-25
26-10 VL seq	81-419	26-138
Linker seq	420-464	139-153
26-10 VH seq	465-821	154-272

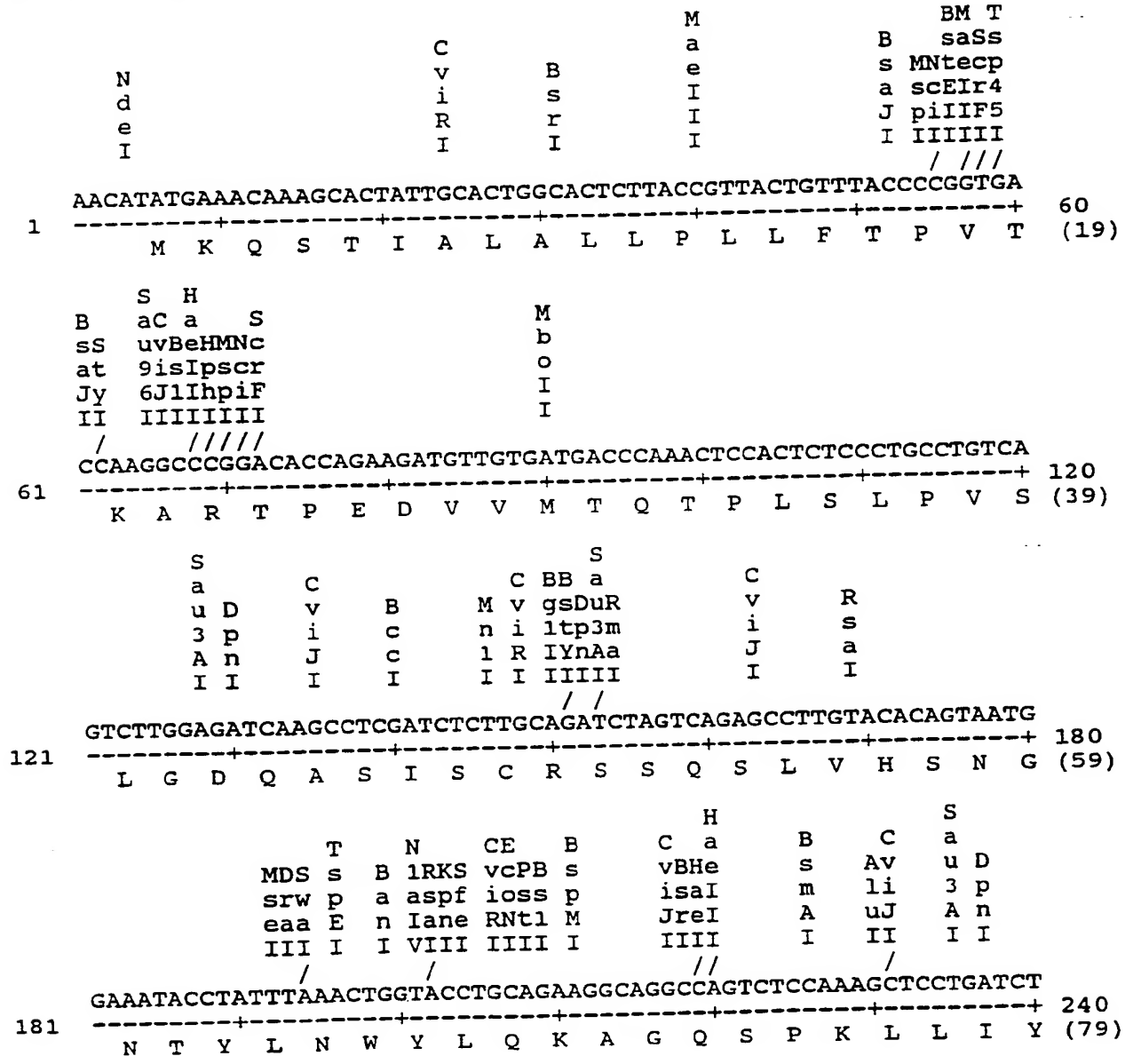


FIGURE 6 (continued)

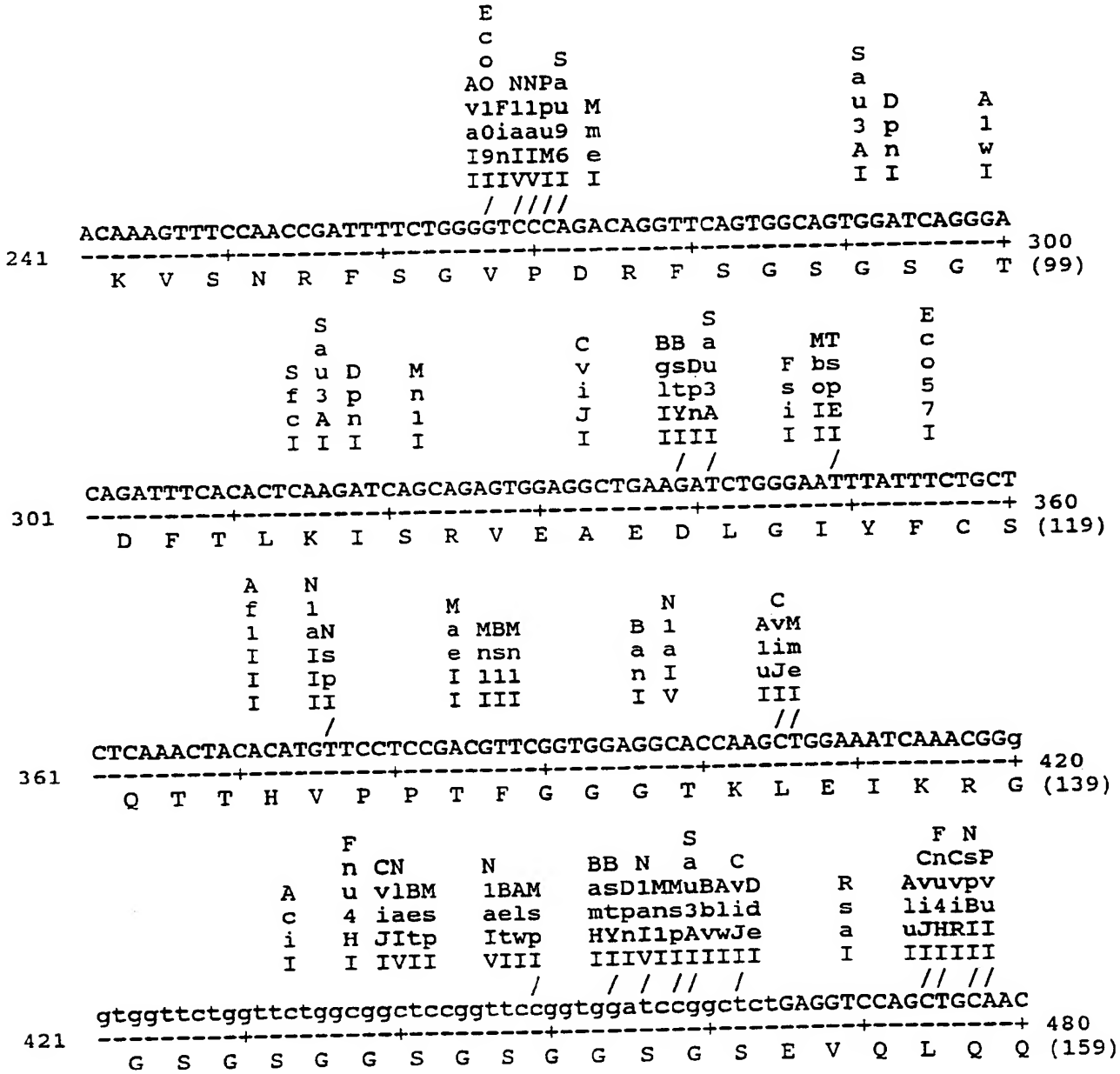


FIGURE 6 (continued)

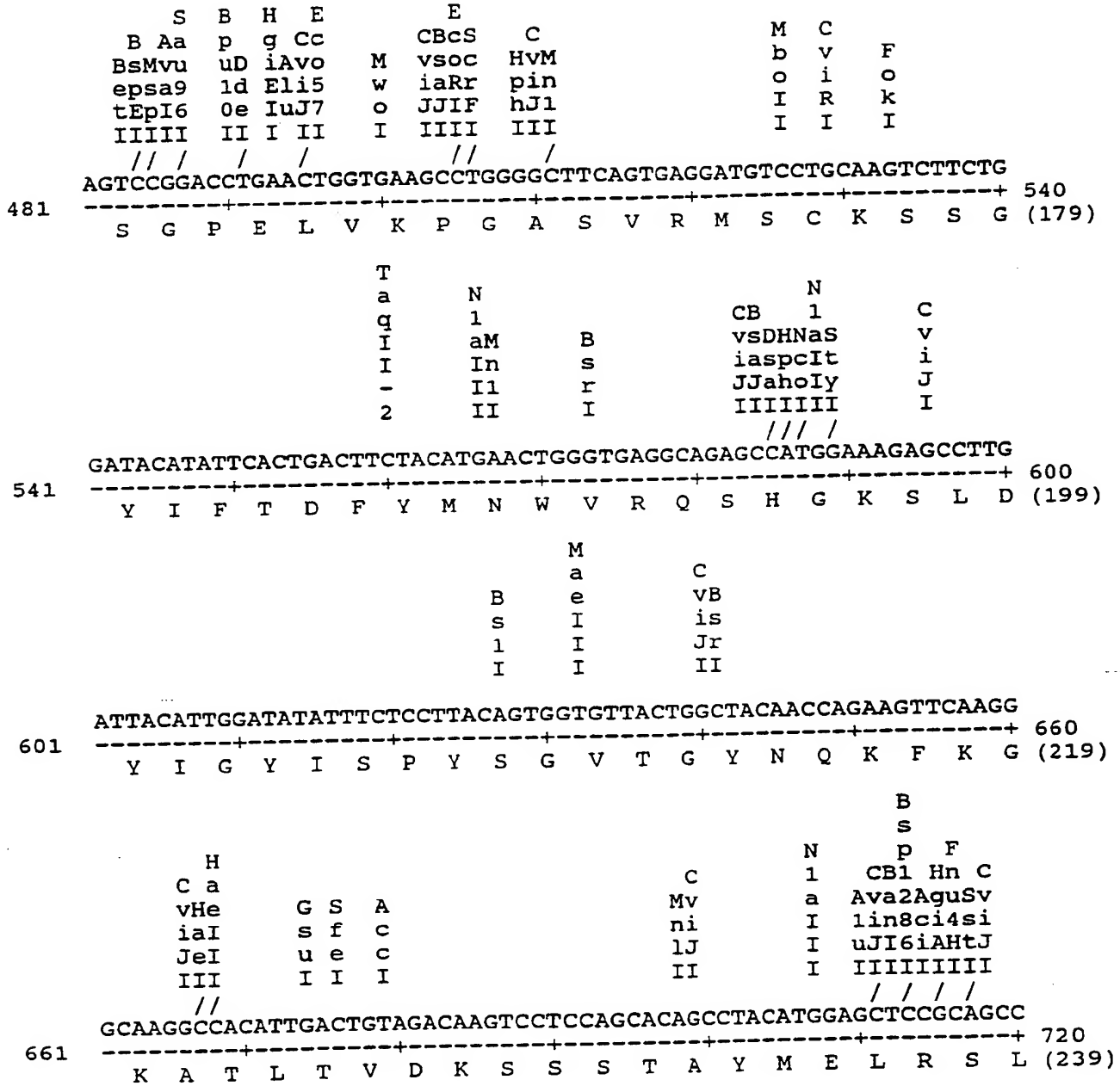
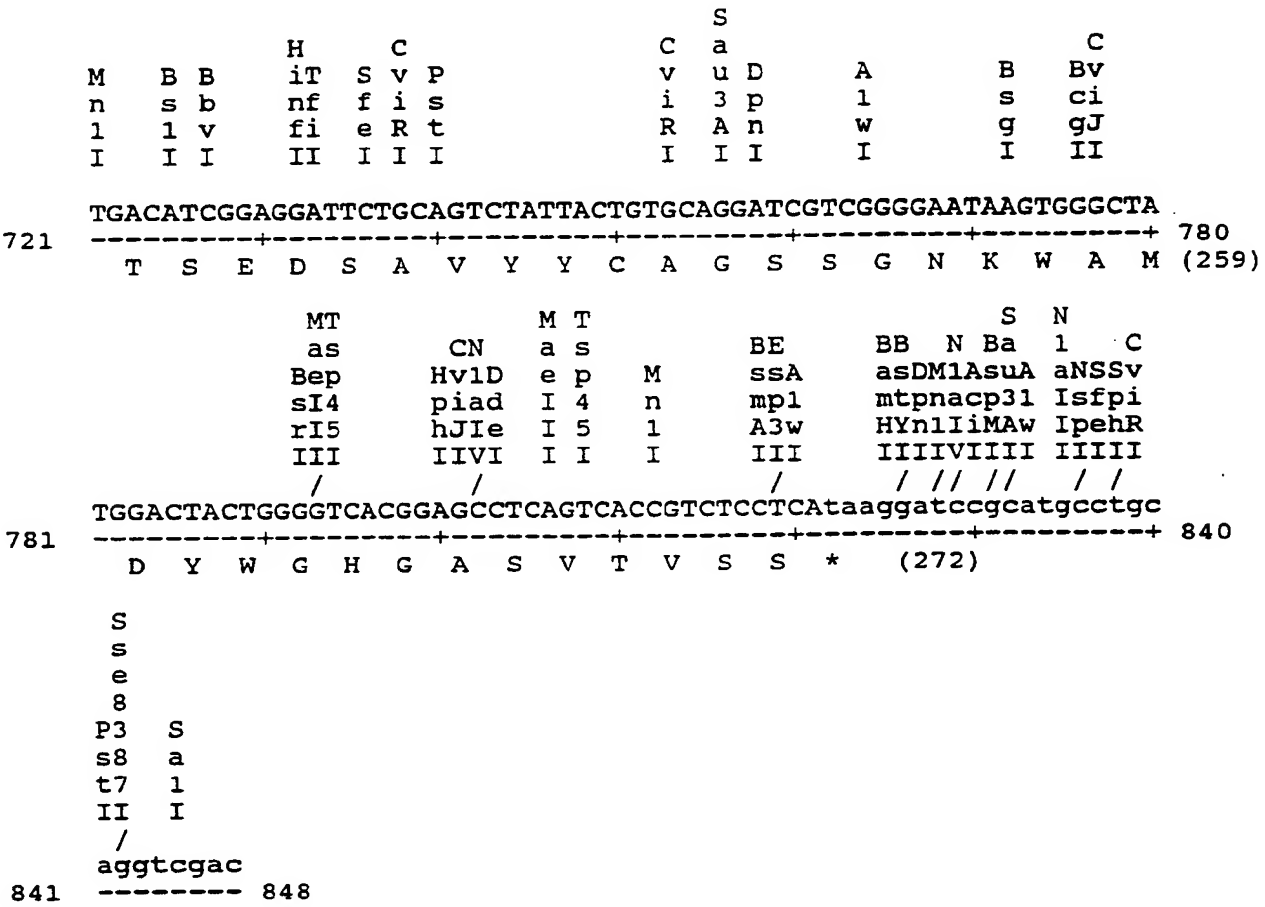


FIGURE 6 (continued)



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US92/08881

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :C12P 21/06
US CL :435/69.6
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : NONE

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

none

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, Dialog, "single chain Fv" "T7 promoter" "phoA" "single peptide sequence"

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Proceedings of the National Academy of Sciences, volume 82, issued February 1985, Tabor et al., "A bacteriophage T7 RNA polymerase/promoter system for controlled exclusive expression of specific genes", pages 1074-1078, see entire article.	4
Y	Science, volume 240, issued 20 May 1988, Skerra et al., "Assembly of a functional immunoglobulin Fv fragment in Escherichia coli", pages 1038-1040, see entire article.	1-6
Y	Methods in Enzymology, volume 185, issued 1990, Studier et al., "Use of T7 RNA polymerase to direct expression of cloned genes", pages 60-89, see entire article.	1-6
Y	Science, volume 242, issued 21 October, 1988, Bird et al., "Single-chain antigen-binding proteins", pages 423-426, see entire article.	1-6
Y	Science, volume 240, issued 20 May 1988, Better et al., Escherichia coli secretion of an active chimeric antibody fragment", pages 1041-1043, see entire article.	1-6

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 10 JANUARY 1993	Date of mailing of the international search report 29 JAN 1993
Name and mailing address of the ISA/ Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. NOT APPLICABLE	Authorized officer SUZANNE ZISKA Telephone No. (703) 308-0196

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